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Filamentous Sulfur Bacteria of Activated Sludge: Characterization of *Thiothrix*, *Beggiatoa*, and Eikelboom Type 021N Strains

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Seventeen strains of filamentous sulfur bacteria were isolated in axenic culture from activated sludge mixed liquor samples and sulfide-gradient enrichment cultures. Isolation procedures involved plating a concentrated inoculum of washed filaments onto media containing sulfide or thiosulfate. The isolates were identified as *Thiothrix* spp., *Beggiatoa* spp., and an organism of uncertain taxonomic status, designated type 021N. All bacteria were gram negative, reduced nitrate, and formed long, multicellular trichomes with internal reserves of sulfur, volutin, and sudanophilic material. *Thiothrix* spp. formed rosettes and gonidia, and four of six strains were ensheathed. Type 021N organisms utilized glucose, lacked a sheath, and differed from *Thiothrix* spp. in several aspects of cellular and cultural morphology. *Beggiatoa* spp. lacked catalase and oxidase, and filaments were motile. Biochemical and physiological characterization of the isolates revealed important distinguishing features between the three groups of bacteria. Strain differences were most evident among the *Thiothrix* cultures. A comparison of the filamentous sulfur bacteria with freshwater strains of *Leucothrix* was made also.

Certain filamentous sulfur bacteria, notably *Thiothrix* and *Beggiatoa* spp. and an unnamed organism, type 021N (11), have been observed in the biomass of aerobic wastewater treatment systems (13, 15, 19, 26, 38, 41). Excessive growth and development of these bacteria may confer upon the normally flocculent activated sludges poor settling and compaction properties, resulting in a condition known as filamentous bulking.

Knowledge of the ecology and taxonomy of wastewater filamentous sulfur bacteria has been hampered by the inability to obtain axenic strains for comparative study. The relationship of type 021N bacteria to *Thiothrix* and *Leucothrix*-like organisms, for which morphological resemblance exists, is poorly understood (5, 11, 29), and the problem of misidentification of these bacteria in activated sludge has been addressed in a recent review (29).

Filamentous microorganisms similar in certain respects to *Leucothrix mucor* were isolated by Poffe et al. (30). The bacteria did not oxidize sulfide or require NaCl and were considered as freshwater *Leucothrix* strains. Isolates of type 021N and *Thiothrix* from activated sludge have not been described previously, although the latter has been isolated from sulfide-containing natural waters (22).

In this study, techniques are described for enrichment and isolation of *Thiothrix*, *Beggiatoa*, and Eikelboom type 021N bacteria from activated sludge, and the salient characteristics which distinguish each group are reported.

MATERIALS AND METHODS

Sample collection and observation of specimens. A total of 20 grab samples of mixed liquor was obtained from aeration basins of 17 activated sludge treatment systems located primarily in Pennsylvania. Approximately 500 ml of mixed liquor was collected at the surface to 0.3 m in depth. Samples were taken personally or by treatment plant attendants, in which case contents were received by mail. The mixed liquors were stored at 10°C and examined microscopically (magnification, ×160 to ×2,000) with the aid of a Zeiss Universal microscope equipped with Neofluar phase-contrast objectives (Carl Zeiss Inc., New York, N.Y.) within 48 h of arrival at the laboratory. Cell dimensions were determined by using a calibrated ocular micrometer at ×2,000 magnification.

Types of filamentous bacteria were observed by microscopic examination of mixed liquor specimens prepared as wet mounts or stained slides and identified on the basis of morphological features in accordance with keys and methods developed by Eikelboom (11), Eikelboom and van Buijsen (13), and Farquhar and Boyle (14). Operating data were obtained for treatment plants at the time of sample collection.

Culture media. All media components were prepared in double, glass-distilled water and autoclaved at 121°C for 15 min unless otherwise noted. Reagent-grade chemicals were used in all experiments. Stock solutions of vitamins, $Na_2S \cdot 9H_2O$, soluble carbohydrates, and alcohols were sterilized by membrane filtration (0.2 μm; Millipore Corp., Bedford, Mass.) and transferred aseptically as required.

Initial isolation experiments were performed by using the culture media given below and containing ingredients at the concentration indicated per liter of the basal salts solution employed in medium I of Eikelboom (11): sodium acetate, 0.15 g, plus $Na_2S \cdot 9H_2O$, 0.187 g (AcS); glucose, 0.15 g (I); $Na_2S \cdot 9H_2O$, 0.187 g (S); and glucose, 0.15 g, plus $Na_2S \cdot 9H_2O$, 0.187 g (GS).

A second mineral salts-vitamin mix (MSV) was formed which contained the following ingredients per liter: $(NH_4)_2SO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; K_2HPO_4, 0.11 g; KH_2PO_4, 0.085 g; $FeCl_3 \cdot 6H_2O$, 0.002 g; EDTA, 0.003 g; and vitamin solution (11), 1 ml.

The following media were used in subsequent isolation experiments: sodium lactate (L); $Na_2S_2O_3 \cdot 5H_2O$ (T); sodium lactate plus $Na_2S_2O_3 \cdot 5H_2O$ (LT); and yeast autoly-

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sate (Albimi Laboratories, Flushing, N.Y.) plus $Na_2S_2O_3 \cdot 5H_2O$ (YT). Sodium lactate, yeast autolysate, and $Na_2S_2O_3 \cdot 5H_2O$ were supplied at 0.5 g/liter of MSV to produce appropriate media.

Other media used in the study were Casitone-glycerol-yeast autolysate (CGY) (10), standard nutrient broth (Difco Laboratories, Detroit, Mich.), and sucrose-Casitone-yeast extract (SCY) (40).

Axenic cultures of bacteria were maintained on LTH broth which contained the following per liter of MSV: sodium lactate, 1.0 g; $Na_2S_2O_3 \cdot 5H_2O$, 0.5 g; and HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [Sigma Chemical Co., St. Louis, Mo.]), 0.01 M.

All media were adjusted with NaOH to pH 7.2 to 7.5. Solid versions of media contained Bacto-Agar (Difco) (12 g/liter) unless otherwise indicated.

Enrichment cultures. Failure to isolate *Thiothrix* strains by direct plating of mixed liquor samples prompted the development of enrichment cultures as sources of inocula. Glucose (1.5 g) and $Na_2S \cdot 9H_2O$ (0.6 g), singly and in combination, were added per liter of sterile stocks to sterile molten agar (20 g/liter) and dispensed in 5-ml amounts to test tubes (20 mm [outer diameter] by 150 mm). Tubes were allowed to solidify overnight. Before inoculation with 1 ml of mixed liquor, solid media in each tube were overlayed with 15 ml of MSV; final pH was 7.2 to 7.5. Cultures were incubated at 22 to 25°C and examined microscopically for evidence of *Thiothrix* trichomes in the surface biomass.

Isolation of bacteria. Isolation of filamentous bacteria was attempted initially by streak plating serial dilutions (10^0 to 10^{-2}) of raw samples or enrichment culture surface films directly onto GS, S, I, AcS, T, L, LT, and SCY agar media. Subsequently, a washing-sonication pretreatment step was employed to increase the density of filamentous bacteria in the inoculum and to reduce the content of adventitious microorganisms. The procedure involved the transfer of several loopfuls of enrichment culture surface film or activated sludge (10^{-1} dilution) samples to small glass petri dishes containing 7 ml of sterile MSV. Approximately 40 to 50 single filaments or rosettes were individually and successively transferred with sterile, glass micropipettes through a series of six to seven washings in fresh MSV while under observation with a stereomicroscope at ×15 to ×45 magnification. The washed inoculum was transferred to 3 ml of MSV and both spread and streak plated onto isolation media. Plates were incubated at 20 to 22°C and examined periodically (×15 to ×45 magnification) for evidence of filamentous colonies. Suspect colonies were transferred three times on primary isolation media and once on SCY and CGY media to ascertain purity.

Bacteria. Axenic bacteria, tentatively identified as *Thiothrix* strains TH1 and TH3, Eikelboom type 021N strains N2, N4, N5, N6, and N7, and *Leucothrix*-like strain N11, were originally isolated and supplied by M. Richard, University of California, Berkeley (M. Richard, G. Shimizu, D. Jenkins, T. Williams, and R. Unz, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q60, p. 270). *Leucothrix*-like strains L1 and L2, described by Poffe et al. (30), were obtained from H. Verachtert, University of Leuven, Belgium. Axenic cultures of all organisms were maintained in LTH broth, stored at 10°C, and subcultured monthly.

Characterization of bacteria. Morphological features were examined on cultures grown on LT or LTH medium at 28°C. Gram reaction (Hucker modification), catalase, and Kovacs oxidase were determined as described by Skerman (33). Volutin and sudanophilic granules were detected with Neisser stain and Sudan black B, respectively (33). Presumptive identification of internal sulfur granules in cultures grown on S and T agars was based upon solubility in 95% ethanol (13), extraction and crystalization with pyridine (33), and general cytological characteristics (14).

Hydrolysis of casein (5 g/liter), gelatin (5 g/liter), starch (5 g/liter), Tween 80 (5 ml/liter), tributyrin (5 ml/liter), and urea (20 g/liter) and dissimilatory nitrate reduction (KNO_3, 0.5 g/liter) were tested as described by Skerman (33) with YT as the basal medium. Indole from tryptophan (0.5 g/liter) and ammonia from arginine (0.5 g/liter) were determined by standard procedures (35) with modified LT (ammonium salt omitted) as the basal medium. Ability to grow anaerobically on LT medium was tested in anaerobic jars with a $CO_2 + H_2$ GasPak generator (BBL Microbiology Systems, Cockeysville, Md.). Growth on complex media was examined with and without $Na_2S_2O_3 \cdot 5H_2O$ (0.25 g/liter). The benzidine dihydrochloride test for iron porphyrins was performed as described by Deibel and Evans (9). Acid from thiosulfate in YT broth was determined by monitoring pH changes. Oxidative-fermentative attack on carbohydrates (0.5 g/liter), prepared in semisolid (0.3% agar) MSV containing 0.016 g of phenol red per liter, was determined by Hugh and Leifson (18). Changes in the pH of liquid culture media were monitored also. Cultures grown on 0.1-strength LT medium exposed to constant illumination (10 days) were tested for the presence of chlorophyll *a* (25) after extraction in 80% (vol/vol) acetone.

All tests were performed in duplicate, and uninoculated media served as negative controls. Positive controls involved inoculation of test media with mixed liquor specimens.

Temperature and pH studies. The ability of strains to develop in LTH broth at various temperatures (see Table 6) was examined. Constant-temperature water baths and incubators were employed. Growth at pH values of 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5 was determined in LTH broth. Tris (0.01 M) was substituted for HEPES buffer in LTH medium at pH values of 8.5, 9.0, and 10.0. Incubation was at 25°C.

Culture development was monitored visually for up to 3 weeks, followed by sonication at 40 W for 15 s (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) and absorbance measurement (400 nm). Tubes were scored according to the extent of turbidity (biomass). Mean absorbance values greater than four times that of the control were considered positive, two to four times control values were scored as marginal, and less than two times the control were regarded as no growth.

Inocula for temperature and pH studies were grown on LTH broth. Cells were washed twice by centrifugation (7,000 × *g* at 10°C for 15 min), suspended in 30 ml of MSV, and dispersed by sonication at 40 W for 20 to 30 s under ice. Duplicate tubes containing 8 ml of media received 0.1 ml of cell suspension.

Electron microscopy. Ultrastructural studies were performed on washed cells from 2- to 3-day-old shake cultures developed on LTH medium at 25°C. Negative staining was accomplished by placing a drop of specimen onto Formvar-carbon-coated 400-mesh grids for 3 to 5 min, draining excess fluid, and staining with 0.5% uranyl acetate for 30 s. Grids were examined with a Philips EM-300 electron microscope at an accelerating voltage of 60 kV.

In preparation for thin sections, cell pellets were pre-fixed in 2.5% glutaraldehyde plus 0.15% ruthenium red in 0.1 M sodium cacodylate buffer (pH 7.3) for 1 h at room temperature. Cells were washed three times in cacodylate buffer and

suspended in 1% OsO_4 and 0.15% ruthenium red in the same buffer for 1 h. The samples were washed three times in double-distilled water and embedded in 2% Noble agar (Difco) contained in glass vials. Small blocks of agar were cut, rinsed in distilled water, and dehydrated through a gradient of ethanol (25, 50, and 75%, 15 min; and 95 and 100%, 30 min). The fixed and dehydrated specimens were passed through an infiltration series of Spurr low-viscosity epoxy resin (36) in ethanol (1:3, 1:1, and 3:1 dilutions, 60 min). The samples were soaked in 100% Spurr resin overnight and polymerized at 70°C for 8 h. The preparations were sectioned with a diamond knife on an LKB Ultratome III ultramicrotome (LKB Instruments, Inc., Rockville, Md.) by using a clearance angle of 4° and a cutting speed of 1 mm/s.

Thin sections (ca. 800 Å [80 nm]) were transferred to 300-mesh, uncoated copper grids and post-stained with 10% uranyl acetate in 50% methanol (15 min) and 0.4% lead citrate (10 min) (31). Grids were rinsed twice with distilled water during final staining.

Antibiotic sensitivity testing. The sensitivity of selected strains of *Thiothrix*, type 021N, and *Leucothrix* to various antibiotics was tested by the multiple dilution approach with Nunc microtiter plates (Vangard International, Neptune, N.J.). Antibiotics (Sigma) were tested in duplicate in LT medium at final concentrations of 0.0, 0.125, 0.25, 0.50, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, and 64.0 mg/liter of the organic moiety (salts excluded). Each well (final volume, 2.5 ml) received 0.1 ml of a standard suspension of washed cells adjusted to an absorbance value of 0.1 at 400 nm. Incubation was at 25°C, and the MIC of antibiotics observed to completely inhibit growth after 4 days was recorded. Immediately before testing, filter-sterilized (0.2 μm) stock solutions of antibiotics were prepared, and 0.1 ml of an appropriate dilution was added to microtiter plates.

RESULTS

Occurrence of filamentous sulfur bacteria in bulking activated sludge. Microscopic examination of bulking mixed liquor solids revealed the presence of three distinct groups of filamentous sulfur bacteria. A total of 17 various morphological types were recognized. The appearance of the sulfur bacteria in samples was as indicated in the following sections on organism characteristics.

Eikelboom type 021N organisms were the fourth most frequently observed bacterial type, appearing in 30% of all samples. Trichomes were very long (>500 μm) and often occurred free in the mixed liquor. The filaments contained unevenly distributed sulfur granules both in situ and upon exposure to 0.1% sodium sulfide.

Thiothrix spp. were sixth in frequency of occurrence, appearing in 20% of activated sludge samples. Two morphological forms of *Thiothrix* spp. similar to those described by Eikelboom and van Buijsen (13) were recognized. The two types differed in filament width and by the presence or absence of a sheath. Epiphytic bacteria were not observed on trichomes of either *Thiothrix* spp. or type 021N organisms. *Beggiatoa* spp. were observed in only 1 of 20 samples; *Thiothrix* spp. were present also.

One sample of bulking solids contained type 021N and both *Thiothrix* types. The aeration basin bulk water dissolved oxygen (DO) level was 6 mg/liter. However, the influent wastewater contained no measurable DO and included an industrial source of carbohydrate and appreciable levels of sulfate and sulfide (10 mg of HS^- per liter). *Thiothrix* filaments were present also.

Enrichment cultures. The diffusion of thiosulfate or sulfide ion from agar butts to overlying mineral salts solution appeared to encourage the development of *Thiothrix* spp. A dense, white tuft of dangling cell mass containing individual trichomes and rosettes appeared in the upper 5 mm of the liquid after 3 to 5 days. The whitish material from several enrichment cultures was pooled and used as the source of *Thiothrix* strains. The morphological characteristics of the trichomes which developed were indistinguishable from those present in the original mixed liquors. Enrichments were maintained for several weeks by the transfer of filamentous biomass to freshly prepared culture tubes.

Isolation of filamentous sulfur bacteria. Attempts to isolate the filamentous sulfur bacteria directly by streak or spread plating were unsuccessful. Small colonies or clusters of filamentous bacteria which developed on plates were difficult to recognize and separate, even under ×15 to ×45 magnification, due to heavy overgrowth by heterogeneous bacteria in the inoculum.

Pretreatment of the inoculum by successive washing greatly reduced the content of organisms responsible for background contamination, and numerous well-separated colonies of the desired filamentous bacteria appeared on plates. The pretreatment step enhanced the recovery of *Thiothrix* spp. from enrichment cultures and type 021N organisms from mixed liquors.

Beggiatoa cultures were obtained by direct plating of enrichment matter and were purified by repeated sectioning and transfer of agar blocks containing the spiral and coiled colonies.

Isolation media, strain designations, and the source of isolated and acquired strains are given in Table 1. Four strains of *Thiothrix*, eight strains of type 021N, and five strains of *Beggiatoa* were obtained in axenic culture. Two of the type 021N isolates were recovered from pet food processing wastewater. The filamentous sulfur bacteria generally developed well on media containing low levels of sole carbon sources with sulfide or thiosulfate but not on complex media (SCY and CGY).

Characteristics of the biological treatment systems from which the bacteria were isolated directly or which served as starting material for enrichment cultures are given in Table 2. Sludge volume index values for the mixed liquor solids, with one exception, exceeded 150 ml of settled solids per g (dry weight) and indicated a tendency towards activated sludge bulking. The mixed liquors had in situ circumneutral pH values and low levels of DO (0.3 to 2.0 mg/liter). Organic loading rates to the activated sludge systems were 0.15 to 0.51 lb (ca. 68.0 to 231.3 g) of biological oxygen demand per day per lb of mixed liquor solids. The mixed liquor suspended solids carried and influent biological oxygen demand concentrations were typical of normal operating conditions. Filamentous microorganisms, other than the types studied, were observed in the samples.

***Thiothrix* strains.** Cellular and colonial features representative of axenic *Thiothrix* cultures are presented in Fig. 1. All strains formed the characteristic rosettes which consisted of filaments radiating outward from foci (Fig. 1a). The aggregation of individual filaments appeared to be aided by the presence of a densely staining, amorphous holdfast located at the center of the rosette (Fig. 1b).

Base-to-tip differentiation along the length of the filaments could be observed (Fig. 1c). Basal cells were typically broader in diameter than the apical gonidia; however, this distinction was not observed for all trichomes, even within the same culture. True knots (loops in trichomes) were observed incidentally.

TABLE 1. Filamentous bacteria isolated from activated sludge wastewater treatment plants

Bacterial group and strain designation[a]	Sampling date (mo/yr)	Isolation medium[b]	Treatment plant location	Wastewater category
Thiothrix spp.				
TH1	8/80	GS	San Jose, Calif.	Domestic/industrial (fruit canning)
TH3	2/81	GS	Courtland, Ala.	Industrial (pulp and paper)
A1[c]; A3[c]	10/81	GS	Aliquippa, Pa.	Domestic
I[c]; Q[c]	12/81	LT	State College, Pa.	Domestic
Type 021N				
N2	11/80	I	Vancouver, Wash.	Industrial (fruit/malt/food process)
N4; N6	12/80	GS	Berkeley, Calif.	Domestic
N5	1/81	GS	Fredonia, N.Y.	Domestic
N7	3/81	GS	Concord, Calif.	Domestic
AP1; AP3	12/81	GS	Aliquippa, Pa.	Domestic
AP4; AP5				
AP9; AP10	12/81	AcS	Aliquippa, Pa.	Domestic
A; B	9/82	LT	Allentown, Pa.	Industrial (pet foods)
Leucothrix sp.				
L1; L2	NA[d]	NA[d]	Belgium	Industrial (petrochemical)
N11	10/81	GS	Jeffersonville, Ind.	Industrial (chemical)
Beggiatoa spp.[c]				
U1	12/81	LT	State College, Pa.	Domestic
SC1; SC4	12/81	AcS	State College, Pa.	Domestic
SC2; SC3	12/81	GS	State College, Pa.	Domestic

[a] Series TH and N strains of *Thiothrix*, type 021N, and *Leucothrix* originally isolated by M. Richard, University of California, Berkeley. *Leucothrix* strains L1 and L2 originally isolated and described by Poffe et al. (30).
[b] Medium composition given in the text.
[c] Strains isolated from enrichment cultures started with filamentous activated sludges.
[d] NA, Not available.

Individual filaments of *Thiothrix* spp. were multicellular with cylindrical cells arranged uniseriately along the length of the trichome. Septa were clearly visible in cells without sulfur granules. Rod-shaped gonidia with rounded ends were typically observed at the apical portion of the filaments or free in the medium. The development of immature rosette clusters may involve the aggregation of freely dispersed gonidia (Fig. 1d), many of which possessed a tuft of subpolar fimbriae (Fig. 1e). Individual gonidia were observed to glide over the surface of glass microscope slides coated with LT medium solidified with 1% agar. The movement was jerky and intermittent, and the cells often displayed a twitching behavior.

Thiothrix strains TH1, TH3, I, and Q developed readily discernible, rigid sheaths (Fig. 1f). Strains A1 and A3 failed to exhibit a clearly defined sheath by electron microscopic examination or lysozyme-detergent treatment (14) and staining with methylene blue. False branching was never observed.

Thiothrix strains grown on LT broth appeared to form two groups on the basis of cell dimensions. Cells of strains A1, A3, and TH3 were 0.7 to 1.5 μm wide and 0.7 to 3.0 μm in length, whereas, cells of strains TH1, I, and Q were of greater overall length (1.2 to 2.5 μm) and width (1.2 to 2.5 μm). Two organisms (strains A1 and A3) appeared distinctive in that they hydrolyzed casein, lacked detectable catalase activity, and required thiosulfate or sulfide for growth. The addition of catalase (20 U/ml; Sigma) to organic media

TABLE 2. Characteristics of activated sludge treatment systems from which filamentous sulfur bacteria were isolated[a]

Bacterial group and strain designation	Treatment plant operating characteristics						Other filamentous microorganisms present[f]
	SVI[b] (ml/g)	F/M[c]	DO (mg/liter)	MLSS[d] (mg/liter)	BOD[e] (mg/liter)	pH	
Type 021N (AP series)	58	0.21	ND[g]	2,729	163	7.1	Type 0041; *Nocardia* spp.; *Sphaerotilus natans*; *Nostocoida limicola*
Type 021N (A; B)	ND	0.15	0.3	3,708	ND	7.3	Type 0041
Thiothrix spp. (A1; A3)[h]	200	0.35	2.0	3,800	350	6.9	Type 1701; type 021N; type 0961; *Nocardia* spp.
Thiothrix spp. (I; Q)[h] and *Beggiatoa* spp. (all strains)[h]	393	0.51	1.5	2,340	369	7.1	Type 1701; type 1863; *Haliscomenobacter hydrossis*

[a] Data presented for those strains isolated in the authors' laboratory only. Source of isolates given in Table 1.
[b] SVI, Sludge volume index (volume of settled solids per gram [dry weight]).
[c] F/M, Organic loading rate (pounds of biological oxygen demand per day per pound of mixed liquor solids).
[d] MLSS, Aeration basin mixed liquor suspended solids concentration.
[e] BOD, Influent wastewater biochemical oxygen demand.
[f] Filament types as described by Eikelboom (11).
[g] No data available.
[h] Strains isolated from enrichment cultures started with mixed liquors.

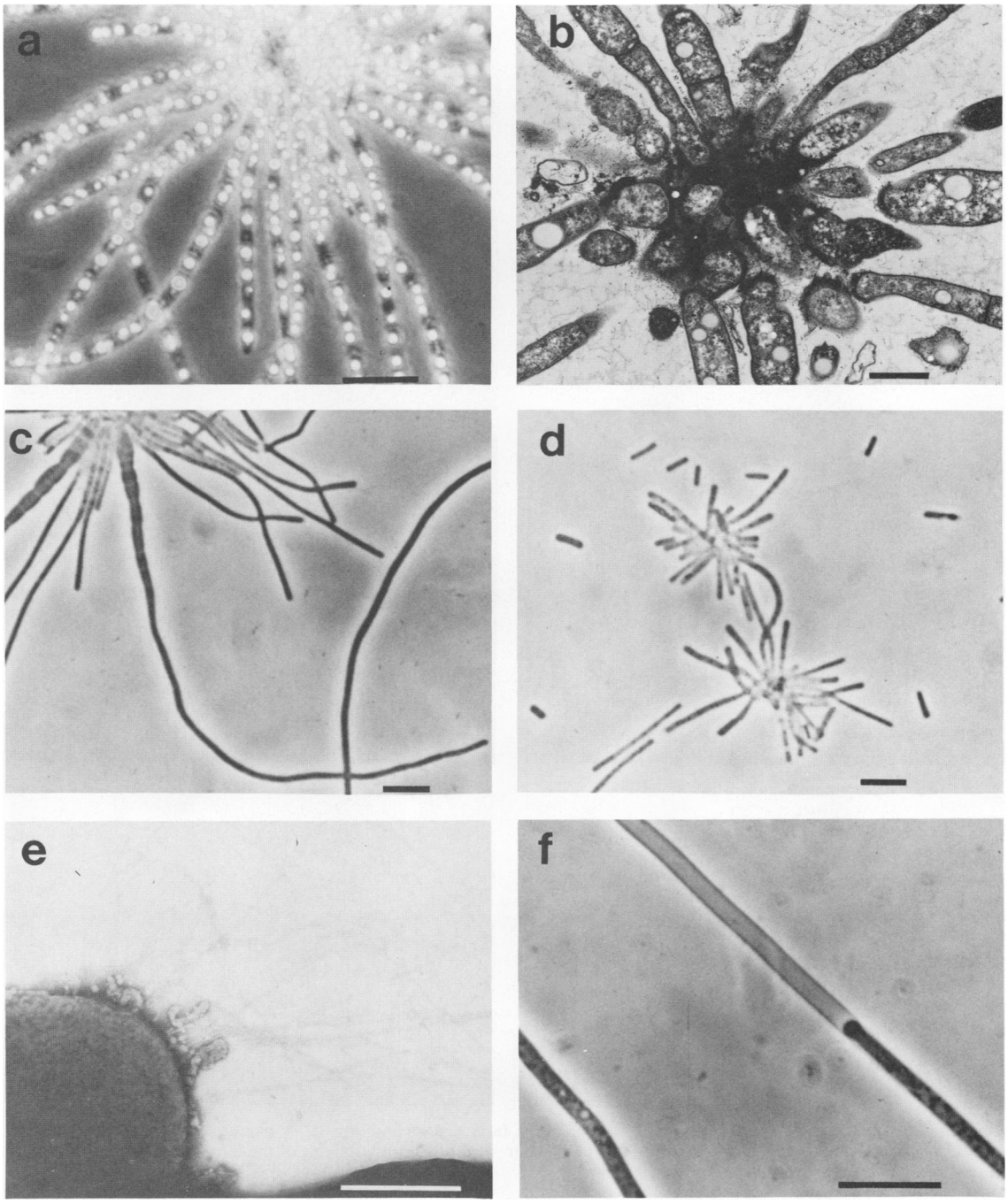

FIG. 1. Light micrographs and transmission electron micrographs showing morphological features of axenic *Thiothrix* strains. (a) Rosette with internal sulfur granules and (b) associated holdfast material (strain A1). (c) Base-to-tip differentiation and (d) free and aggregated gonidia (strain I). (e) Gonidia with tuft of fimbriae (strain A1). (f) Sheath material (strain Q). (a, c, d, and f) Wet mount; phase contrast; bar = 10 μm. (b) Thin section; bar = 2 μm; and (e) negatively stained; bar = 0.5 μm.

containing lactate, acetate, glucose, fructose, or pyruvate as sole carbon sources failed to encourage growth in the absence of thiosulfate or sulfide.

Sulfur granules (Fig. 1a) were observed in strains grown on liquid or solid media containing sulfide or thiosulfate but not sulfite. The size and extent of the granules varied with the isolate and state of growth and were more pronounced generally in media containing reduced amounts of organic matter, e.g., S and T agar or LTH broth modified by reducing sodium lactate from 1.0 g/liter to 75 mg/liter. Sulfur granules within cells were extracted with pyridine, and the sulfur formed rhombic crystals external to the filaments. Treatment with ethanol resulted in compact granules being released intact. The granules were refractile and displayed concentric rings of yellow, red-violet, and blue coloration when viewed by phase-contrast microscopy.

Transformation of reduced sulfur compounds in YT broth resulted in acid generation and lowering of the pH from 7.3

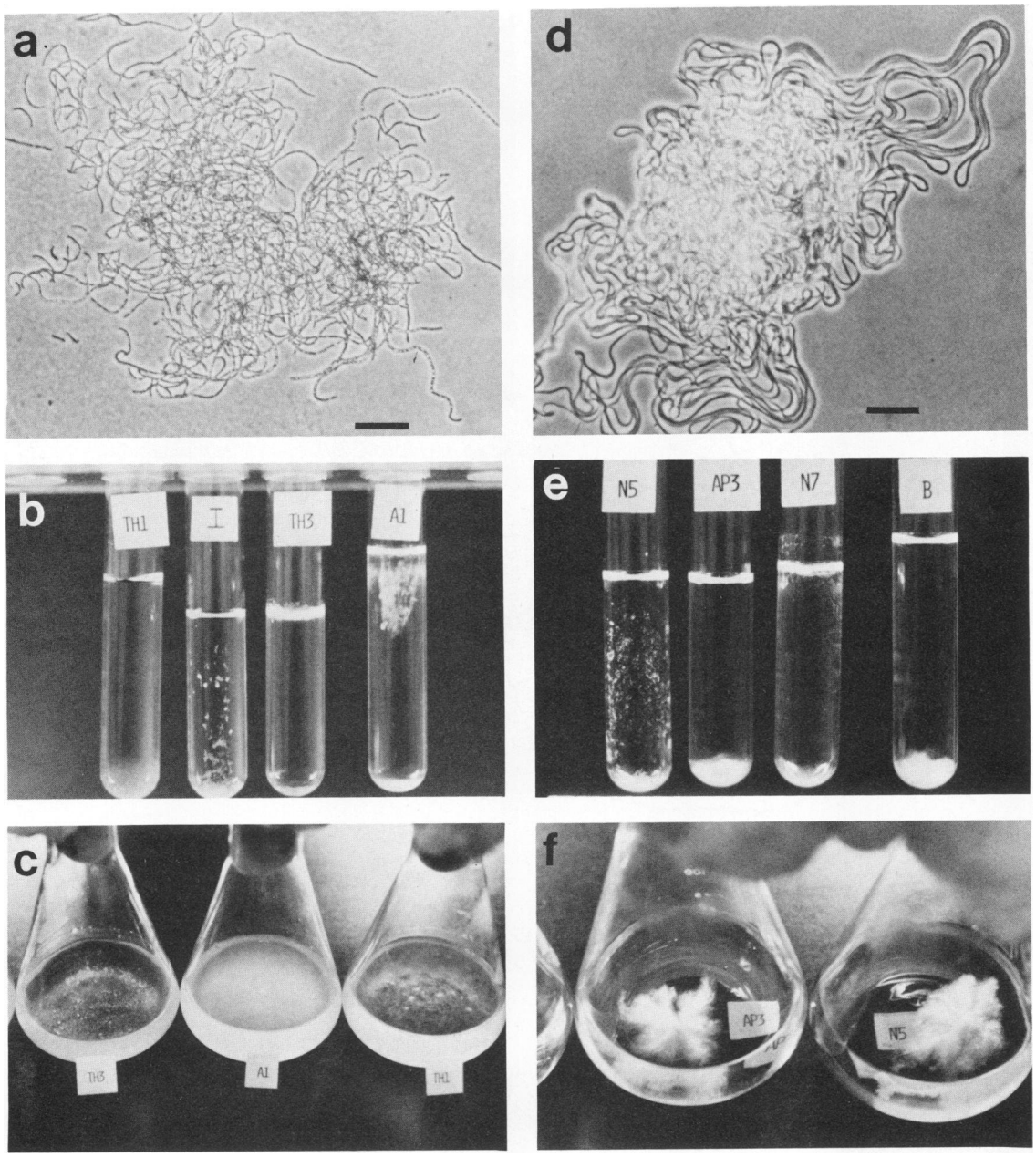

FIG. 2. Growth habit and cultural characteristics of *Thiothrix* spp. (a, b, and c) and type 021N bacteria (d, e, and f). Colony morphology of *Thiothrix* strain A1 (a) and type 021N strain B (d) on LTH agar; bar = 30 μM. Growth habit of the bacteria in shaken (b and e) and static (c and f) cultures.

to 5.8 to 6.5. Sodium sulfide (0.2 g/liter) and sodium thiosulfate (0.5 g/liter) served as sole sources of sulfur for the *Thiothrix* isolates when tested in liquid L medium (chloride salts substituted for sulfate salts).

Isolates grown on LT agar developed visible colonies after 2 to 3 days of incubation at 28°C. Colonies were less than 2 mm in diameter, white to off-white in color, hard to the touch, and flat to slightly raised; they also exhibited rough to slightly wavy or thread-like margins (Fig. 2a). When cultured on T or S agar, colonies viewed by reflected light developed blackened centers after 5 to 7 days, presumably owing to sulfur transformation.

The growth habit of *Thiothrix* cultures was observed to vary according to culture conditions. In shaken cultures, the biomass ranged from primarily turbid to flocculent, depending on the isolate (Fig. 2b). Turbid cultures consisted of short (<200 μm) filaments, freely dispersed gonidia and small immature rosettes. Flocculent masses appeared to be largely due to clumping and entanglement of filaments and rosettes.

The development of strains under static incubation (Fig. 2c) ranged from primarily a firm, granular surface pellicle (strain TH3), which readily settled out upon disturbance, to a light, fluffy, and dangling mat-like surface mass (strains A1 and A3) reminiscent of enrichment cultures. Wall growth was common. Filament length was noticeably greater (>500 μm) in static cultures, and filaments were often entangled and rope-like in appearance.

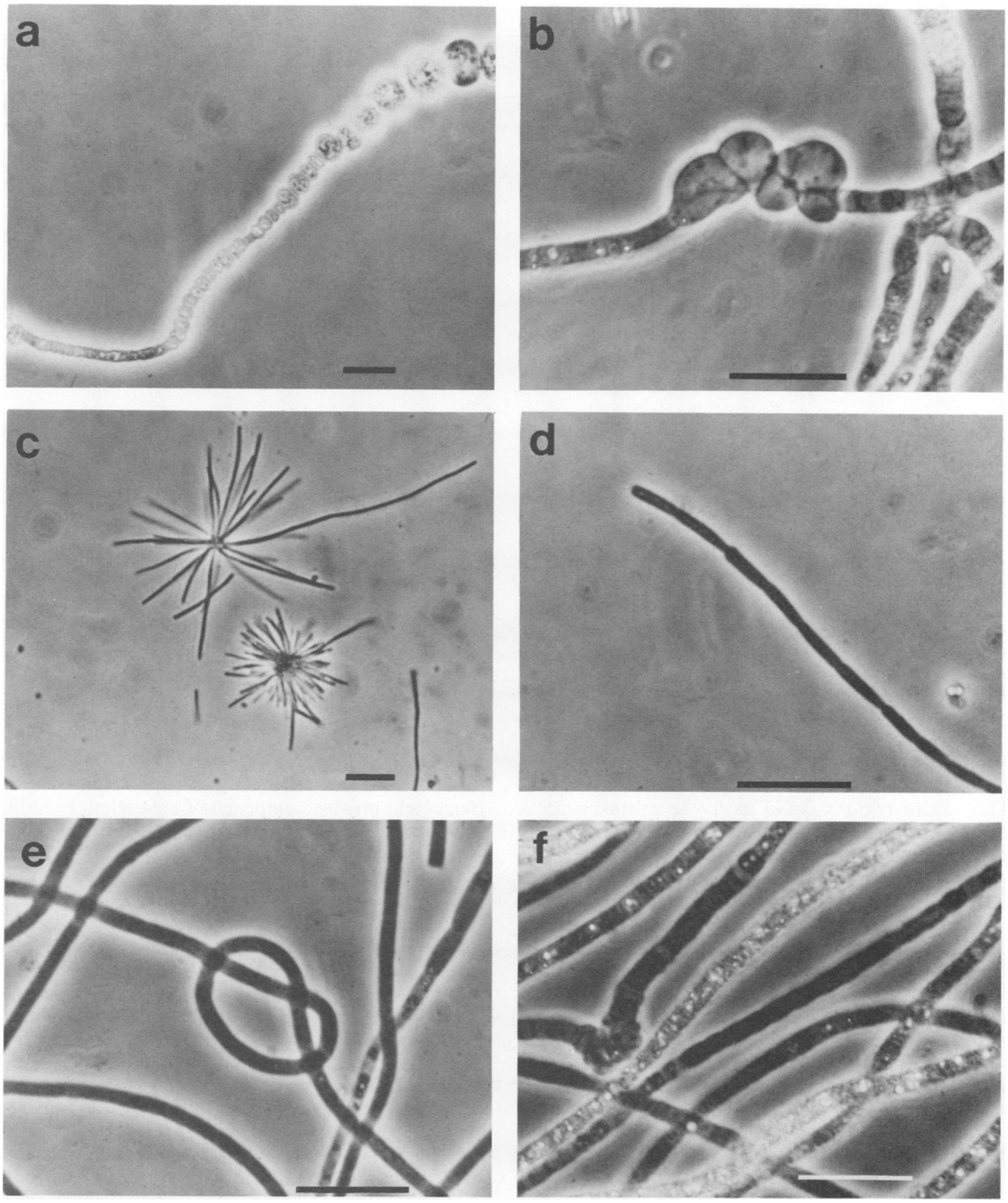

FIG. 3. Morphological features of Eikelboom type 021N bacteria in pure culture. (a) Discoid to bead-like cells with (b) swollen regions (bulbs) (strain AP3). (c) Rosettes and (d) apical gonidia (strain B). (e) True knot and (f) internal sulfur granules (strain AP4). Wet mount; phase contrast; bar = 10 μm.

The development of *Thiothrix* cultures in semisolid (0.3% agar) LTH medium revealed an aerobic to microaerophilic preference for oxygen. Growth was most dense initially within the upper 3 mm of the culture tubes and was visible to a depth of 20 to 37 mm below the surface. Slight banding below the surface was observed for certain strains after 3 weeks of incubation.

Type 021N strains. Type 021N bacteria formed long, multicellular trichomes with readily visible septa. Individual filaments displayed much variation in cell morphology, with cuboidal to barrel-shaped or cylindrical cells typical, although certain regions contained largely discoid or bead-like cells (Fig. 3a). Cell dimensions of strains grown on LT broth ranged from 1.0 to 3.0 μm in width by 1.0 to 5.0 μm in length. Strain differences in morphology were evident. Clusters of large, swollen cells (bulbs) located along the length of filaments (Fig. 3b) were observed. Large vacant areas within filaments or empty "cuffs" at the tips were common, resulting possibly from cell lysis or mechanical breakage of cells during the preparation of specimens for microscopic obser-

TABLE 3. Growth of filamentous bacteria at various temperatures and pH values[a]

Group and strain designation	Growth at:											
	Temp (°C)[b]						pH[c]					
	4	10	20–28	33	37	45	5.0	6.0	6.5–8.0	8.5	9.0	10.0
Thiothrix spp.												
TH1	−	+/−	+	+	+/−	−	−	−	+	+/−	−	−
TH3	−	+/−	+	+	+/−	−	−	+	+	+	−	−
A1	+	+	+	+	−	−	−	−	+	+	−	−
A3	+	+	+	+	−	−	−	−	+	+	−	−
I	+/−	+	+	−	−	−	−	−	+	+	−	−
Q	+/−	+	+	−	−	−	−	−	+	+	−	−
Type 021N												
N2	−	+	+	+	+	−	−	+	+	+/−	−	−
N4	+/−	+	+	+	+/−	−	−	+	+	+/−	−	−
N5	−	+	+	+	−	−	−	−	+	−	−	−
N6	+/−	+	+	+	+	−	−	+	+	+/−	−	−
N7	−	+/−	+	+	−	−	−	−	+	−	−	−
AP3	−	+	+	+	−	−	−	−	+	+/−	−	−
AP4	−	+	+	+	+/−	−	−	+/−	+	+	−	−
A	−	+	+	+	+/−	−	−	+/−	+	+/−	−	−
B	−	+	+	+	+/−	−	−	+	+	+/−	−	−
Leucothrix sp.												
L1	−	+	+	+	−	−	−	−	+	+	+	−
L2	−	+	+	+	−	−	−	−	+	+	+	−
N11	−	+	+	+	−	−	−	−	+	+	+	−
Beggiatoa spp.												
U1	−	+	+	+/−	−	−	−	−	+	−	−	−

[a] Culture development determined from absorbance readings of sonicated cultures. Vigorous growth (+) equals greater than four times control absorbance values; slight or marginal growth (+/−) equals two to four times control absorbance values; no growth (−) equals less than two times control absorbance values.
[b] LTH medium; incubation for 1 to 3 weeks.
[c] LTH medium buffered as described in text; incubation for 1 to 2 weeks.

vation. The vacant regions could easily be mistaken for a sheath, despite the presence of visibly intact cell septa. A true sheath was never observed.

Certain but not all type 021N organisms formed rosettes (Fig. 3c). However, only those strains capable of producing rosettes developed apical gonidia (Fig. 3d). These structures were not observed in cultures of other strains, regardless of the incubation temperature (4 to 37°C), medium pH (6.0 to 8.5), and strength (0.1 to 1.0 g of sodium lactate per liter). Rosettes were similar in appearance to those described for *Thiothrix* spp. in that a densely staining amorphous holdfast was present, gonidia (rod- to oval-shaped) were fimbriated, and tapering of filaments occurred. True knots (Fig. 3e) may be present.

Sulfur granules (Fig. 3F) were formed by all strains and appeared visibly abundant in cultures developed on S or T agar and modified LT broth. The extent to which type 021N bacteria lowered pH during growth in YT broth varied among strains, and final pH values ranged from 6.4 to 6.7. Growth in yeast autolysate medium alone resulted in neutral to slightly alkaline (pH 7.3 to 7.7) reactions. Both sulfide and thiosulfate served as sole sulfur sources for type 021N strains N2, N5, AP4, and B. Other cultures were not examined.

Growth of type 021N on solid media was visible after ca. 48 h at 28°C. Colonies appeared thumbprint-like or woven and were 2 to 4 mm in diameter, off-white to white in color, stringy, sticky to the touch, and flat to slightly raised with wavy to curled margins (Fig. 2d). Colonies on S or T agar formed blackened centers after 5 to 7 days, as viewed by reflected light.

Agitated cultures of type 021N organisms contained large flocculent masses composed of entangled, rope-like clusters of filaments, often star-like in appearance (Fig. 2f). Turbidity was typically absent.

Two major growth forms were apparent in static cultures (Fig. 2e). Rosette-forming strains, e.g., strain N5, often developed as a network of interconnected filaments and rosettes throughout the culture and were frequently attached to the sides or bottoms of tubes. Surface growth occurred occasionally, but heavy turbidity was not observed. Non-rosette-forming strains occurred almost exclusively as a light, fluffy, cohesive mat of interwoven filaments of great length (>1 mm) which remained at the base of tubes and could be removed intact with an inoculating loop.

Growth in soft agar deeps was most abundant within the upper 3 to 4 mm of the tubes, indicating a preference for aerobic conditions. Slight growth occurred to a depth of 20 to 25 mm, and little or no banding was observed.

Temperature and pH studies. The development of filamentous bacteria at various temperatures and pH values is compared in Table 3. All organisms were capable of vigorous growth at 20 to 28°C and pH 6.5 to 8.0, but none could proliferate at 45°C or at pH 5.0 and 10.0. Important differences within and among the groups were evident. Certain *Thiothrix* strains developed at 4°C, whereas others were more tolerant of higher temperatures (33 to 37°C). Most isolates grew at pH 8.5 but not pH 6.0. Type 021N strains which grew at pH 6.0 were less able to develop at pH 8.5. Certain strains grew marginally at 37°C. The *Leucothrix* isolates were most similar in temperature and pH tolerance; all strains grew at pH 9.0, but not pH 6.0 or at 4 or 37°C. The *Beggiatoa* culture displayed a narrow range of tolerance to pH and temperature. In addition, cultures which developed

TABLE 4. Sensitivity to antibiotics by strains of *Thiothrix*, type 021N, and *Leucothrix*

Compound tested	Primary spectrum[b]	MIC[a] with:				
		Thiothrix spp.		Type 021N		*Leucothrix* sp.
		A1	TH1	AP4	N2	N11
Streptomycin	Broad	<0.125	<0.125	<0.125	<0.125	<0.125
Gentamicin	Broad	<0.125	<0.125	<0.125	<0.125	<0.125
Tetracycline	Broad	0.25	<0.125	0.25	<0.125	<0.125
Ampicillin	Broad	0.25	<0.125	0.25	1.0	<0.125
Penicillin G	Gr^+	2.0	<0.125	4.0	2.0	<0.125
Polymyxin B	Gr^-	0.5	0.5	2.0	2.0	ND
Chloramphenicol	Broad	2.0	2.0	1.0	1.0	0.5
Bacitracin	Gr^+	64.0	>64.0	16.0	32.0	4.0
Sulfanilamide	Broad	0.25	>64.0	>64.0	>64.0	>64.0
Lincomycin	Gr^+	>64.0	>64.0	>64.0	>64.0	>64.0

[a] Values given as milligrams per liter of the organic moiety observed to completely inhibit growth within 4 days of incubation. Compounds tested in liquid LT medium at 25°C. ND, No data available.
[b] Gr^-, Gram-negative; Gr^+, gram-positive.

at lower temperatures were typically ropy or clumped and very cohesive, whereas a more dispersed and flocculent biomass which was easily fragmented with moderate agitation formed at 33 to 37°C.

Antibiotic sensitivity. The effect of various antibiotics on selected strains of *Thiothrix*, type 021N, and *Leucothrix* is presented in Table 4. All bacteria were completely inhibited by the broad-spectrum compounds streptomycin and gentamicin (MIC of <0.125 mg/liter) and were, with the exception of *Thiothrix* strain A1, resistant (MIC of >64 mg/liter) to lincomycin and sulfanilamide at the levels tested. Moderate sensitivity to polymyxin and chloramphenicol was noted.

TABLE 5. Comparison of salient features of filamentous sulfur bacteria

Characteristic	% Positive characteristics with:			
	Thiothrix spp. (6)[a]	Type 021N (13)	*Leucothrix* sp. (3)	*Beggiatoa* spp. (5)
Gram-negative	100	100	100	100
Volutin reserves	100	100	100	100
Sudanophilic granules	100	100	100	100
Nitrate reduced to nitrite	100	100	100	100
Iron porphyrins	100	100	100	0
Oxidase present	100	100	100	0
Rosettes formed	100	38	67	0
Gonidia present	100	38	67	0
Catalase present	67	8	100	0
Glucose utilized	17	100	100	0 (1)
Sulfur granules	100	100	0	100
Acid from thiosulfate	100	100	0	0 (1)
Gelatin hydrolysis	83	100	0	0
Colonies hard	100	0	0	0
Sheath present	67	0	0	0
Casein hydrolysis	33	0	0	0
Sulfur required[b]	33	0	0	0
Urea hydrolysis	17	0	0	0
Acid from carbohydrates[c]	17	0 (9)	0	0
Filaments motile	0	0	0	100
Acid from alcohols[c]	0	0 (9)	0	100 (1)
Growth at pH 9	0	0	100	0 (1)
Growth on nutrient agar	0	0	100	0
Denitrification	0	0	0	0 (1)
Tween 80 hydrolysis	0	0	0	0
Starch hydrolysis	0	0	0	0
Anaerobic growth	0	0	0	0
Indole from tryptophan	0	0 (9)	0	0 (1)
Ammonia from arginine	0	0 (9)	0	0 (1)

[a] Number of strains tested except where indicated within parentheses in columns.
[b] Strains required thiosulfate or sulfide for growth.
[c] Strong acid reaction (pH <5.5) produced from selected carbohydrates and alcohols (Table 6).

TABLE 6. Acid reaction by strains of filamentous bacteria cultured on carbohydrates and alcohols[a]

Compound[b]	Mean final pH for:																
	Thiothrix spp.				Type 021N									*Leucothrix* sp.			*Beggiatoa* spp.
	TH1	I	Q	TH3	N2	N4	N5	N6	N7	AP3	AP4	A	B	N11	L1	L2	U1
Carbohydrates																	
Glucose	5.0	—	—	—	6.2	6.4	6.4	6.0	6.0	6.0	6.2	6.7	6.7	6.8	6.1	6.5	—
Fructose	5.2	5.9	5.9	—	6.0	6.4	5.8	6.2	6.2	6.0	6.3	6.5	6.4	6.7	6.4	6.6	—
Maltose	4.8	—	—	—	6.0	6.4	6.1	6.1	6.1	6.3	6.3	6.5	6.5	6.6	6.3	6.6	—
Sucrose	4.8	6.0	6.1	—	5.7	6.6	6.3	6.2	6.0	6.2	6.2	6.5	6.6	6.8	5.8	6.6	—
Trehalose	5.3	—	—	—	5.9	6.5	6.4	6.2	6.0	6.4	6.4	6.5	6.4	6.7	6.4	6.6	—
Melezitose	6.0	6.0	6.1	—	6.1	6.5	6.4	6.2	5.9	6.4	6.3	6.5	6.5	6.6	6.5	6.6	—
Raffinose	—	—	—	—	—	—	—	6.0	—	—	—	—	—	—	—	—	—
Mannitol	5.4	—	—	—	6.2	6.5	6.5	6.3	5.9	6.3	6.3	6.7	—	6.7	6.5	6.6	—
Inositol	5.9	—	—	—	—	6.4	6.2	6.1	—	—	6.3	—	—	6.6	6.4	6.5	—
Primary alcohols																	
Ethanol	6.5	—	—	6.6	—	—	—	6.6	6.4	—	—	—	—	—	—	—	4.5
Propanol	6.5	—	—	6.0	—	—	—	—	6.2	—	—	—	—	—	—	—	4.5
Butanol	6.2	—	—	—	6.5	—	—	—	6.3	—	—	—	—	—	—	—	4.6
No substrate added (control)	6.6	6.9	6.9	6.8	6.7	6.8	6.9	6.7	6.9	6.8	6.8	7.1	7.0	6.9	7.0	6.9	6.9

[a] Only those strains which demonstrated growth on any of the above compounds are shown. Incubation period was 1 to 3 weeks at 22 to 25°C. —, No growth on the compound tested.

[b] Substrates tested at 0.5 g/liter in MSV; initial pH was 7.1 to 7.4.

Type 021N strains responded similarly as a group to the antibiotics tested. The *Leucothrix* isolate, in comparison to type 021N and *Thiothrix*, was more sensitive to the antibiotics penicillin and bacitracin which were generally active against gram-positive bacteria.

Comparative features of filamentous sulfur bacteria. Salient features of the filamentous sulfur bacteria are summarized in Table 5. Results were clustered to aid recognition of key characteristics considered important for each group. Isolates tentatively identified as freshwater *Leucothrix* strains were included for comparison with *Thiothrix* and type 021N strains. All groups of bacteria were gram negative, reduced nitrate to nitrite, and contained sudanophilic granules (presumably poly-β-hydroxybutyric acid) and volutin reserves in aged cells. The sulfur bacteria, in contrast to *Leucothrix* strains, produced internal sulfur granules when grown in the presence of thiosulfate and sulfide, and were unable to develop on full- or 0.1-strength complex media (with or without thiosulfate added) and at pH 9. All *Leucothrix* isolates were catalase and oxidase positive also.

All *Thiothrix* strains formed rosettes and hard or tough colonies, and four of six were ensheathed. Only one strain utilized glucose. Type 021N organisms hydrolyzed gelatin, developed sticky or stringy colonies on plating media, and, with one exception, were catalase negative. *Beggiatoa* filaments exhibited gliding motility and lacked oxidase, catalase, and iron porphyrins.

No strains were capable of growth with thiosulfate or sulfide as sole energy sources with or without bicarbonate present. Negative characteristics for all strains included anaerobic growth and denitrification (Table 5). The bacteria were nonsporulating, nonflagellated, nonpigmented, and exhibited no measurable evidence of chlorophyll *a* production.

A comparison of acid generated from carbohydrates and alcohols revealed important differences among the bacteria. Acid production, when observed, occurred oxidatively, and no gas was evident. A strong acid reaction (final pH, <5.5) from carbohydrates was observed for one of three *Thiothrix* isolates capable of growth on any of the substrates tested (Table 6). Final pH values observed on carbohydrate media were 4.8 (maltose and sucrose), 5.0 (glucose), and 5.4 (mannitol). Type 021N and *Leucothrix* cultures utilized most of the carbohydrates tested; however, only slight (final pH, 5.6 to 6.0) or no change in pH occurred. The *Beggiatoa* isolate failed to develop on any sugars but produced a strong acid reaction (final pH, 4.5 to 4.6) from ethanol, propanol, and butanol.

The three *Leucothrix* strains examined were unsheathed and formed long (>500 μm) filaments consisting of barrel- to oval-shaped cells 1.5 to 2.5 μm in width and 1.0 to 2.5 μm in length. Cultural characteristics of the bacteria on solid and liquid media were similar in most respects to those described for type 021N organisms.

The *Beggiatoa* isolates formed trichomes of variable length and contained cylindrical cells which were 1.2 to 1.5 μm in width and 2.0 to 6.0 μm in length. Colonies were flat, white to off-white in color, highly coiled, and spreading. Agitated broth cultures were ropy and slightly turbid and displayed tuft-like aggregates. Subsurface bands of cell mass appeared in soft agar deeps, indicating a tendency towards microaerophilic behavior.

DISCUSSION

Wagner (41) and Eikelboom (11) reported type 021N to be a predominant filamentous bacterium in bulking sludges. Strom and Jenkins (38) observed type 021N, *Thiothrix* spp., and *Beggiatoa* spp. in 33, 7, and 2% of mixed liquor samples, respectively. These values agree well with the results obtained in this study.

Specific operating parameters considered to be important to the development of the sulfur bacteria in activated sludge include low DO (13, 38), increased organic loading (12), or sulfide-containing (septic) wastewaters (16, 26, 29, 38). Acknowledging direct cause-and-effect relationships between crude wastewater parameters and the growth of specific filamentous bacteria should be undertaken with caution, since undefinable interactions involving environmental variables and the complex wastewater community metabolism

may be the true stimulus of filamentous bacterial activity. We observed *Thiothrix* spp. and type 021N bacteria under conditions of both low and high aeration basin DO levels with influent sulfides present. Two of our *Thiothrix* strains and those of Larkin and Shinabarger (23) required sulfide or thiosulfate for growth. Reduced sulfur compounds may be required to promote the development of similar bacteria in the field. The elimination of sulfides has been considered an important measure for controlling *Thiothrix* bulking (16, 26); however, Pipes (29) noted that septic wastewaters may deliver large numbers of *Thiothrix* bacteria to the treatment facility.

Previous investigators have reported difficulty in isolating *Thiothrix* and *Thiothrix*-like bacteria from activated sludge (14, 40) and natural waters (2, 6, 20), a problem possibly resulting from the use of inappropriate isolation procedures and culture media. In this study, plating of washed filaments onto media containing low levels of single organic substrates and thiosulfate or sulfide proved successful for the recovery of axenic cultures of *Thiothrix*, type 021N, and *Beggiatoa* strains from mixed liquors and enrichment cultures. Extensive washing of individual filaments and rosettes greatly reduced background contamination which aided in the isolation of filamentous bacteria. Larkin (22) recently obtained pure cultures of *Thiothrix* strains from sulfur springs by employing a version of micromanipulation and media containing 0.03% or less added carbon and 0.03% sodium sulfide. Similar types of media low in organic content with reduced sulfur sources have been used for the isolation of *Beggiatoa* strains (28, 37). Complex media used for the isolation of *Sphaerotilus natans* and other filamentous bacteria (10, 14, 30) failed to support growth of the sulfur bacteria.

The genus *Thiothrix* currently comprises eleven species (4, 24, 32), several of which are of questionable validity (4, 21, 24). Only *T. nivea*, the type species, is currently recognized (4, 34). Larkin and Shinabarger (23) characterized two strains of *T. nivea* which (i) formed sulfur granules; (ii) produced gliding gonidia, rosettes, and a sheath; (iii) were oxidase positive and catalase negative; (iv) lacked caseinase and gelatinase; and (v) appeared as obligate mixotrophs. The isolates befit the original description of the genus *Thiothrix* by Winogradsky (42), and strain JP2 (ATCC 35100) was designated the neotype of *T. nivea*.

The *Thiothrix* strains described herein may be placed within the genus *Thiothrix*; however, important differences from *T. nivea* exist. Four of the six strains examined were catalase positive and did not require sulfide for growth. The two remaining strains were obligate mixotrophs but lacked an apparent sheath and possessed caseinase and gelatinase. Given the above taxonomic considerations and the diversity exhibited among the collected *Thiothrix* strains with regard to cell width, temperature tolerance, reaction to carbohydrates, and antibiotic sensitivity, assignment of species epithets was not presently considered.

The taxonomic status of Eikelboom type 021N bacteria is presently unclear. Eikelboom (11) initially considered type 021N bacteria to be identical to the *Leucothrix* sp. of Cyrus and Sladka (8) and two of the growth forms of *Thiothrix* spp. described by Farquhar and Boyle (15). The generic epithets *Leucothrix* and *Thiothrix* were considered inappropriate for type 021N, however, in that the former appeared obligated to the marine environment (3) and the latter was characterized by the regular presence of large sulfur globules. Eikelboom (12) acknowledged the presence of *Thiothrix* spp. in a later report and subsequently described two growth forms (13). *Leucothrix* spp. have since been reported several times in activated sludge (7, 30). Poffe et al. (30) isolated cultures similar in certain respects to *L. mucor* (3, 5) from a petrochemical wastewater treatment plant and considered them to be freshwater strains of *Leucothrix*.

Our type 021N strains resembled *Thiothrix* spp. in that both formed long, nonmotile trichomes and deposited sulfur granules internally. Unlike *Thiothrix* spp., type 021N bacteria utilized glucose and other carbohydrates, produced no sheath, and exhibited more pronounced variability in cell morphology. Rosettes and gonidia were observed in only 5 of 13 strains. Colony morphology and overall growth habit most closely resembled the freshwater *Leucothrix* strains. However, unlike type 021N bacteria, *Leucothrix* strains were catalase positive, developed on complex media, grew at pH 9, and did not form sulfur granules.

Type 021N strains appeared to share characteristics of both genera *Thiothrix* and *Leucothrix*, and a new genus designation to accommodate type 021N bacteria is warranted.

Thiothrix spp. occur in nature primarily in flowing waters containing sulfide (4, 24). Their presence has been reported in cool (10°C) (2, 27) and warm (38 to 40°C) (21, 39) springs, shallow wells (21), irrigation systems (17), drainage ditches (20), and lakes (6, 32). Waters were typically circumneutral (pH 6.7 to 7.3) (1, 20, 27) to slightly alkaline (21) in pH. Laboratory studies on axenic *Thiothrix* cultures (Table 3) demonstrated the potential to develop within the above temperature and pH limits, although certain strains would be less likely to survive the higher temperatures.

Aside from waste treatment systems, the distribution of type 021N bacteria in the natural environment is not known. Habitats suitable for *Thiothrix* spp. may be exploited by these organisms, and the possibility exists that type 021N organisms may have been mistakenly identified as growth forms of *Thiothrix* spp. in nature.

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